Decadent Sporulation Mutants of *Bacillus subtilis*

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In decadent sporulation mutants, sporulating populations are heterogeneous: the cells reach successive chemical and physical resistances with progressively decreasing frequencies. Each decadent mutant can be characterized by the shape and slope of the curve describing the frequency of cells resistant to various agents ('the resistance spectrum'). In some mutants the resistance spectrum decreases progressively from xylene resistance to heat resistance; in other mutants it decreases rapidly between octanol resistance and chloroform resistance. Electron microscopy showed that in two mutants the majority of the cells are blocked at stages III and IV; the number of cells that develop further to reach successive morphological stages falls off progressively. In two other mutants most cells reach stage V. Cortexless spores are also frequent. One of the decadent mutations, *SpoLI*, was localized between *aroD* and *acf*. The phenotype of decadent mutants is discussed in terms of sequential gene activation.

INTRODUCTION

Sporulation mutants can be characterized in general by the last morphological stage reached by the sporulating cells. Mutants blocked at any of the morphological stages from 0 to VI have been found (reviewed by Balassa, 1971; Piggot & Coote, 1976). Normally the block is clearly defined: the majority of the cells reach a given stage, and no cells undergo further development. [Cells in which sporulation stops earlier than this stage are, however, frequently present (see Balassa & Yamamoto, 1970).] In a study of the sequential development of chemical resistances in sporulating cells, a peculiar mutant (DB16) was found in which the majority of the cells reached only stage III or IV, but rare cells reached octanol resistance and even chloroform resistance with frequencies of about 10^{-2} and 10^{-4} , respectively (Balassa & Yamamoto, 1970; Balassa *et al.*, 1974). Apparently in this mutant successive steps of spore formation are reached with decreasing frequencies. Such a phenotype will be called 'decadent'. ('Decadence: the process of...declining, decay, impaired condition' – *Shorter Oxford English Dictionary*. This term seems more appropriate than the previously used term 'attenuation'.) Recognition of decadent mutants is based on the stepwise decrease in the frequency of spores resistant to the successive chemical treatments. The purpose of this paper is to present a detailed morphological, physiological and genetic study of mutant DB16. In addition eight other, newly described decadent mutants are characterized phenotypically.

Sporulation genes are under a common genetic control and their expression is submitted to one-directional pleiotropy: each gene controls the activation of all the 'later' but none

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of the 'earlier' genes (Balassa, 1969, 1971; Balassa & Yamamoto, 1970; Piggot & Coote, 1976). We believe that the decadent phenotype is due to some type of regulatory mutation and reflects an alteration in the mechanism of sequential gene activation.

METHODS

Strains. Strain DB16 (Institut Pasteur Culture Collection no. 7627) was obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutagenesis of germinated spores (Balassa, 1969). Strains FA10 (IPCC no. 7628), FD10 (IPCC no. 7629) and FE9 (IPCC no. 7630) were obtained in a similar manner (J. Zucca & G. Balassa, unpublished). Strains SE10, 11, 12, 13 and 16 were isolated as late sporulation mutants from ultraviolet-irradiated spores of the original Marburg (SMY) strain (Millet & Ryter, 1972).

Sporulation. This was achieved in nutrient broth as described previously (Balassa, 1969; Dod *et al.*, 1978). Time t_0 was defined as the end of exponential growth; t_1 , t_2 etc. indicate sporulation times in hours after t_0 .

Chemical treatments. These were done as described by Milhaud & Balassa (1973). Undiluted samples of sporulating cultures, at t_{24} unless otherwise specified, were treated for 15 min at room temperature with 0.1 vol. of organic solvents [90% (v/v) ethanol was used]. Trichloroacetic acid (TCA) resistance was measured after incubating the cells at 4 °C with 5% (w/v) TCA. Heat resistance was measured by incubating samples of cultures at 80 °C for 10 min.

Genetic methods. Transformation and transduction (by phage PBS1) were done as described by Milhaud *et al.* (1978). The chloroform replica technique consisted of replicating agar plates with velvet before and after exposure to chloroform vapour (30 min) and identifying the colonies present on the first replica but absent from the second after incubation.

Electron microscopy. Cells were sampled at the times indicated, fixed according to the Ryter & Kellenberger (1958) procedure and embedded in Epon as described by Sousa *et al.* (1974). Thin sections were photographed and at least 100 sporulating cells were examined in each section. The morphological stages II (membrane invagination), III (enclosed forespore), IV (cortex), V (coat development) and VI (mature spore) were identified according to Ryter (1965) and Balassa (1971). Both longitudinal and transverse sections were counted (Sousa *et al.*, 1978). The frequency of cells at each morphological stage was calculated as the percentage among the total number of sporulating cells; cells which did not show any sign of spore formation were excluded from the calculation (Sousa *et al.*, 1978).

RESULTS

Phenotypic characterization of decadent mutants

Spectrum of chemical resistances

In cells which sporulate normally, resistances to various chemicals develop sequentially, before heat resistance, in the order: xylene, toluene, octanol, chloroform (Milhaud & Balassa, 1973). Thus earlier stages of spore development can be associated with 'early resistances' and later stages with 'late resistances'. Treatment of samples from sporulating cultures with various agents allows one to measure the number of the forespores which have reached a given stage of spore formation. Such 'resistance spectra' were obtained at t_{24} for cultures of nine decadent mutants, using five organic solvents and heat treatment. Although the actual survival values varied considerably from one experiment to another (Table 1), this did not alter the order in which the frequency of cells resistant to different agents decreased or the general shape of the resistance spectra.

For each mutant a typical resistance spectrum was obtained by calculating the geometrical mean (antilogarithm of the mean of the logarithms of the survivals values) of at least two, and in general five to eleven, experiments (Fig. 1). The nine mutants exhibited a variety of resistance spectra. In most of them the decadence, i.e. the decrease in the frequency of survival from one resistance to the next, was progressive, but it occurred at different rates; it was fast in SE12, where no cells became octanol-resistant, and slow in SE11, where cells became heat-resistant at a frequency of 10^{-3} . In DB16 and FD10, however, there was a sudden decrease in frequency between octanol resistance and chloroform resistance. Thus each mutation defines genetically the resistance spectrum of the strain.

Table 1. *Intermediate chemical resistances of strain DB16*

Sporulating cultures were treated at t_{24} with the chemicals indicated, or heated to 80 °C for 10 min. The results indicate the number of cells ml⁻¹ surviving.

Treatment	Expt 1	Expt 2	Expt 3
Octanol	2.9×10^7	4×10^6	5×10^6
Butanol	3.6×10^6	1.5×10^5	2.4×10^6
Methanol	3.7×10^5	7×10^3	3×10^4
Ethanol	3.2×10^5	7×10^3	3×10^4
Acetone	4×10^5	8.5×10^3	1×10^4
Dioxan	7.5×10^4	2.5×10^3	4×10^3
Chloroform	6.3×10^4	5×10^2	4×10^2
TCA	5.2×10^4	ND	3×10^2
80 °C, 10 min	2×10^3	6×10^2	6×10^2

ND, Not determined.

Fig. 1. Resistant spectra of decadent mutants. Samples of sporulating cultures at t_{24} were tested for resistance to xylene (Xy), toluene (Tl), benzene (Be), octanol (Oc), chloroform (Ch) and to heating at 80 °C for 10 min (T); V denotes results for viable cells. The numbers of cells surviving each treatment (geometrical mean values) are shown. The distances on the horizontal axes are proportional to the time of appearance of the resistances in wild-type cells (Milhaud & Balassa, 1973).

In addition to the chemicals mentioned in Fig. 1, normal sporulating cells become progressively resistant to other organic solvents and acids (Milhaud & Balassa, 1973). Thus resistance to butanol appears after octanol resistance, resistances to chloroform, methanol, ethanol, acetone and dioxan appear at about the same time, while TCA resistance and heat resistance appear later. Resistance to these agents was measured in three experiments with DB16 (Table 1). Butanol resistance closely followed octanol resistance. The frequencies of cells resistant to methanol, ethanol, acetone and dioxan were similar, being intermediate between the frequencies of octanol-resistant and chloroform-resistant cells. The number of cells surviving TCA treatment was between the numbers surviving chloroform and heat treatments. These observations confirm the order of the resistances obtained by kinetic experiments with the wild-type strain. Although sporulation of decadent mutants might be slower than sporulation of the wild type (see below), this is not the reason for the low frequencies of resistant cells at t_{24} : the frequency of heat-resistant spores was the same in t_{24} and t_{48} samples of four decadent mutants tested (DB16, FA10, FD10 and FE9).

Sporulation is frequently temperature-sensitive, particularly among oligosporogenous mutants, and few cells sporulate at high incubation temperatures. This was not the case for

Table 2. *Frequency of morphological stages in sporulating cultures of decadent mutants*

The frequencies are expressed as percentages of the total number of sporulating cells. In each sample, 200 or more cells were counted. Stage VI cells include both enclosed and free, apparently mature, spores. Results for strain 168, which sporulates normally, are included for comparison.

Strain	Time of sampling	Percentage of cells in sporulation	Percentage of cells at stage:				
			II	III	IV	V	VI
DB16	t_6	33	3	84	13	0	0
	t_{10}	30	7	41	37	15*	0
FA10	t_{24}	69	1	8	3	59	30
FD10	t_6	44	1	62	36	2*	0
FE9	t_{24}	74	2	6	4	58	31
168	t_6	96	0	1	3	27	68
	t_{20}	92	0	0	1	2	84

* Abnormal early stage V (see text).

DB16 and FE9, as the same resistance spectra were obtained when these mutants were incubated at 30, 37 and 45 °C.

Dipicolinic acid (DPA), a late sporulation product presumably related to heat resistance (see Gould & Dring, 1974; Balassa *et al.*, 1979), was synthesized only in low amounts: t_{24} cultures of DB16 and FE9 contained 8 and 6 $\mu\text{g DPA ml}^{-1}$, respectively, compared with 40 $\mu\text{g ml}^{-1}$ in wild-type cultures. Addition of DPA (100 $\mu\text{g ml}^{-1}$) to sporulating cultures of decadent mutants at t_0 or t_3 did not change their resistance spectra.

The production of the sporulation-associated extracellular protease was measured as described by Dod *et al.* (1978). Two of the decadent mutants, DB16 and FD10, had normal protease activities: 0.10 and 0.13 units ml^{-1} , respectively, at t_{24} , compared with 0.10 units ml^{-1} in *Spo*⁺ cultures. A third strain, FE9, overproduced protease due to the presence of an independent spore control mutation, *Sco15* (Milhaud *et al.*, 1978).

Morphological characterization

Chemical resistances can be used to characterize the small fraction of the population in cultures of decadent mutants which reach the latest stage of sporulation. In contrast, electron microscopy shows the stages reached by a large number of cells. The sporulation of four decadent mutants was studied morphologically in samples taken at t_6 , t_{10} and t_{24} (Table 2). At t_6 , most of the cells in cultures of a *Spo*⁺ strain (168) had reached stage VI, whereas the populations of the four decadent mutants remained heterogeneous. In DB16, the most typical cells showed stage III forespores surrounded by an abnormally thick germ cell wall and enclosed in a lysed sporangium (Fig. 2). Only one-third of the cells reached stage IV and even less attained stage V. In the latter, both the cortex and the coats appeared to be incomplete and immature. For instance, the typical lamellar structure of the inner spore coat was missing (Figs 3, 4). This early stage V, characterized by apparently abnormal assembly of the cortex and of the spore coats, is indicated as 'stage V' in the quantitative studies (Table 2, Fig. 9). (It is possible that the cortex and the coats were present in DB16 cells more frequently than observed but were rapidly degraded.) Cells of FD10 showed the same characteristics as DB16.

In contrast, FA10 and FE9 cells generally reached stage V and frequently stage VI (Figs 5 to 7). Both the inner and outer spore coats were well developed and apparently mature. The ribosomes of the spore cytoplasm remained clearly visible even in stage VI spores, as they are in the chloroform-resistant but heat-sensitive spores of a *dpa* mutant but not in DPA-containing heat-resistant spores (Balassa *et al.*, 1979). Moreover, the cortex was

Bar markers represent $0.5\ \mu\text{m}$ in Figs 2 and 3 and $0.2\ \mu\text{m}$ in Figs 4 to 7.

Figs 2 to 7. Thin sections of spores of decadent mutants sampled at t_{32} (strain DB16) or t_{24} (strain FE9): CW, germ cell wall; CX, cortex or cortex region; IC, inner spore coat; OC, outer spore coat; EX, exosporium.

Fig. 2. Strain DB16, stage III forespore surrounded by a thick germ cell wall. The sporangium is completely lysed. The unlabelled arrow indicates lysis of the sporangial cell wall.

Fig. 3. Strain DB16, early stage V. An incomplete cortex and abnormal, immature spore coat (unlabelled arrows) are present. The sporangium is partially lysed.

Fig. 4. Strain DB16, abnormal stage V. This is the most advanced stage reached by this strain. An incomplete coat is present (unlabelled arrows). Between the thick germ cell wall and the immature coat some cortex-like material can be seen.

Fig. 5. Strain FE9, late stage V. The cortex is reduced to an irregular layer of granular material. The irregular outline of the spore coats suggests that they have been assembled in the absence of a well developed cortex.

Fig. 6. Strain FE9, stage VI. The cortex is missing; the empty areas suggest that it was disorganized and lysed after coat assembly. The coats appear to be mature but highly convoluted. (Transverse section.)

Fig. 7. Strain FE9, stage VI. The coats appear to be mature but highly convoluted as in Fig. 6. The cortex is missing; in some places some granular material (incomplete or partially destroyed cortex?) can be seen. (Transverse section.)

Fig. 8. Development of chemical resistances in (a) strain DB16 (—), (b) strains FA10 (—) and FD10 (····) and (c) strain FE9 (—). Data for control *Spo*⁺ cultures (----) are included in (b) and (c). Results show the numbers of cells resistant to xylene (●), toluene (■), octanol (▲), chloroform (○) and heat, 80 °C for 10 min (□).

Fig. 9. Morphological development of (a) strain 168, wild type (from Sousa *et al.*, 1978) and (b) strain DB16. The numbers of cells at stages II (■), III (○), IV (□), V (●) and VI (▲) are expressed as percentages of the total number of sporulating cells. With strain DB16 (b) 'stage V' cells are blocked at early stage V (see text).

abnormal or missing completely: in an FA10 culture, 70% of the cells which reached stage V were cortex-less at t_{24} . This abnormal appearance might well be due to the degradation of a pre-existing but unstable cortex. The spore coats in these cortex-less spores were convoluted.

No free spores or forespores were found in cultures of the decadent mutants. Apparently sporulation-dependent lysis of the sporangial cell wall did not take place.

Kinetics of development of decadent mutants

The development of the chemical resistances was followed in sporulating cultures of four decadent mutants (Fig. 8). All the resistances appeared later in the mutants and the number of resistant cells increased more slowly than in control *Spo*⁺ cultures. Moreover, the delay was longer and the increase was slower in the most decadent mutants (i.e. those

Table 3. *Genetic mapping of SpoL1 in three-factor crosses*The donor was strain AA32 (*hisB2 SpoL1*).

Genotype of recipient	Selected marker	No. of transductants selected	Percentage cotransfer*
<i>metC3 pheA1 pyrA1</i> (PM12)	Phe ⁺	449	14
	Ura ⁺	400	0
<i>leu-8 pheA trpC2</i> (PM14)	Phe ⁺	281	17.5
	Leu ⁺	229	2
	Trp ⁺	350	0
<i>argA11 lys-1 metC3</i> (PM40)	Lys ⁺	224	4
	Arg ⁺	300	0
<i>acf aroD trpC2</i> (PM49)	AroD ⁺	344	91
<i>acf pheA trpC2</i> (PM47)	Phe ⁺	583	15

* Calculated as $100 \times (\text{no. of Pig}^- \text{ colonies}) / (\text{no. of transductants selected})$.

in which the final frequency of resistant cells was lowest) and with the last resistances reached. To summarize, the lower the final frequency of spores resistant to a treatment, the later this resistance appeared and the slower the number of resistant cells increased.

Morphogenesis was also followed in a DB16 culture by quantitative electron microscopy. Compared with the development of wild-type cultures, all the morphological stages appeared later and persisted for a longer period (Fig. 9). This was particularly obvious for stage III cells which accumulated from t_4 to t_7 and remained present beyond t_{10} . The number of cells which reached stages IV and V increased slowly from t_4 and t_7 , respectively. These results again suggest that in decadent mutants the switch from one morphological stage to the next is delayed and occurs with a low probability.

Genetic studies of decadent mutants

Genetic homogeneity of the populations

Decadent mutants were recognized because their spores reached 'early chemical resistances' more frequently than 'late resistances' or heat resistance. Population heterogeneity of the sporulating cultures might have been due to genetic heterogeneity. However, this was not the case since clones isolated from DB16 colonies obtained by plating untreated cultures (clones from 'viable cells') and clones originated from heat-resistant spores and therefore derived from the small fraction of cells which reached late stages ('spore clones') behaved in the same way and exhibited the same spectrum of resistances as the original strain. The same was true for clones obtained from 'viable cell counts' or from 'octanol resistance counts' of mutants FD10 and FE9. The resistance spectrum of sporulating cultures is therefore genetically determined and decadent mutations introduce phenotypic heterogeneity into genetically homogeneous populations.

Mapping of the SpoL1 mutation

Colonies of DB16 are recognized by their white colour (Pig⁻). In addition, young colonies (15 h incubation) are killed by chloroform vapour and so can be identified by the chloroform replica technique (Ch^s phenotype). These characteristics permitted a genetic study of this decadent mutation. The Pig⁻Ch^s phenotype was introduced by transformation into the *Spo*⁺ strain PM7 (*hisB2 metC3*). Among 369 His⁺ and the same number of Met⁺ transformants selected, 24 and 25 colonies, respectively, were Pig⁻Ch^s. This indicated a frequency of double transformation of 6.7%, comparable with the frequency of double

Table 4. *Genetic mapping of SpoL1 in three-factor crosses*
The donor was strain AA32 (*SpoL hisB2 acf⁺ aroD⁺*).

Genotype of recipient	Selected marker	Transductant colonies			Percentage cotransfer
		Class	No.		
<i>SpoL⁺ acf aroD trpC2</i> (PM49)	AroD ⁺	AroD ⁺ Acf ^R Spo ⁺	29		
		AroD ⁺ Acf ^R SpoL1	38		<i>aroD-SpoL1</i> 85
		AroD ⁺ Acf ⁺ SpoL1	139		<i>aroD-acf</i> 68
		AroD ⁺ Acf ⁺ Spo ⁺	2		
<i>SpoL⁺ acf pheA trpC2</i> (PM47)	Phe ⁺	Phe ⁺ Acf ^R Spo ⁺	111		
		Phe ⁺ Acf ^R SpoL1	1		<i>phe-SpoL1</i> 15.5
		Phe ⁺ Acf ⁺ Spo ⁺	57		<i>phe-acf</i> 44
		Phe ⁺ Acf ⁺ SpoL1	30		

Table 5. *Linkage between SpoL1 and aroD by transformation*

The recipient strain PM49 (*aroD trpC2*) was transformed with either spontaneously excreted DNA or purified DNA (see Milhaud *et al.*, 1978) of AA32 (*hisB2 SpoL1*). For excreted DNA, *N* indicates the DNA concentration of the undiluted supernatant, corresponding to 1.25×10^7 donor cells. In a control experiment, the cotransfer frequencies of *hisB2* with *trpC2* were 54 and 33 % with excreted DNA and purified DNA, respectively. No cotransfer was found between *trpC2* and *SpoL1* ($< 2\%$).

Selected marker	Transformation with excreted DNA			Transformation with purified DNA		
	Concn	No. of colonies selected	Percentage cotransfer of <i>SpoL1</i>	Concn ($\mu\text{g ml}^{-1}$)	No. of colonies selected	Percentage cotransfer of <i>SpoL1</i>
AroD ⁺	N/100	305	23	85×10^{-2}	421	13
AroD ⁺	N/1000	96	19	85×10^{-3}	73	15

Fig. 10. Genetic location of the *SpoLI* locus. The figures indicate the percentage cotransfer frequencies.

transformants for the two independent markers *hisB2* and *metC3*. Control experiments showed that the transformants exhibited a decadent phenotype similar to DB16. Thus the Pig-Ch^s phenotype is apparently due to a single mutation, called *SpoLI*.* One of the transformants, AA32 (*SpoLI hisB2*), was used in further genetic studies.

Transduction experiments with phage PBS1 indicated that three loci *pheA1*, *lys-I* and *aroD* were linked to *SpoLI* with cotransfer frequencies of 16, 4 and 91%, respectively (Table 3). The same results were obtained whether *SpoLI* colonies were identified by their colour or by their sensitivity to chloroform vapour. The decadent phenotype was confirmed with a few selected transductants. Thus, the *SpoLI* gene is apparently situated about half-way between *pheA* and *lys*, close to *aroD*. Since the two known loci situated between *pheA* and *lys*, namely *aroD* (Hoch & Nester, 1973) and *acf* (Ionesco *et al.*, 1970), have not previously been precisely mapped, three-factor crosses were performed both to order these genes and to map *SpoLI* with respect to them (Table 4). Assuming that among the selected Aro⁺ and Phe⁺ transductants the least frequent class corresponds to the double recombinants, the following gene orders were deduced: *aroD-SpoL-acf* (from the cross with PM49) and *SpoL-acf-pheA* (from the cross with PM47). Figure 10 shows the genetic map deduced from these transductions.

As suggested by the high cotransfer frequency between *SpoLI* and *aroD*, the two markers are also linked by transformation. Using saturating concentrations of DNA, with PM49 (*aroD Spo*⁺) as recipient and AA32 (*aroD*⁺ *SpoLI*) as donor, 55% of the colonies selected as Aro⁺ were also *SpoLI*. In the reciprocal transformation, with AA32 as the recipient strain, His⁺ transformants were first selected, and Spo⁺ colonies were identified among them; 40% of these were also Aro⁺. Since high DNA concentrations allow cotransformation of unlinked markers, transformation was repeated with limiting DNA concentrations. Similar results were obtained with dilutions of spontaneously excreted DNA and with purified DNA. The linkage between *SpoLI* and *aroD* was maintained with decreasing DNA concentrations (Table 5). The recombination frequency between the two markers was 83%, compared with a recombination frequency between *trpC2* and *hisB2* of 44%. No linkage was detected by transformation between *SpoLI* and *acf*.

Transformation experiments showed that FE9, the only decadent mutant which overproduces extracellular protease, carries two independent mutations: *SpoL2*, responsible for the decadent phenotype, and *Sco15*, a control mutation leading to protease overproduction (see also Dod *et al.*, 1978). Neither these loci nor the other decadent mutations have been mapped.

* Sporulation mutations are generally designated by *Spo* followed by a roman numeral indicating the stage of the block and a capital letter for the locus, e.g. *SpoVE* for a stage V mutation (Young & Wilson, 1972). Since decadent mutants are heterogeneous with respect to the morphological block, the letter *L* for 'late' is used.

DISCUSSION

Decadent sporulation mutants are characterized by progressively declining frequencies of the sequentially appearing chemical resistances. Each resistance appears in populations of sporulating cultures with a genetically determined frequency. The various decadent mutants differ in the shape and slope of their resistance spectra. The order in which the frequencies of chemical resistances decrease follows the order of appearance of chemical resistances in sporulating cultures of wild-type strains (Milhaud & Balassa, 1973) and confirms the sequential development of spore resistances.

The morphological studies confirm the existence of the 'decadent' phenotype: sporulating cells reach the successive morphological stages with decreasing frequencies. In some mutants, the majority of the cells remain blocked at stages III or IV; in others, many cells reach stages V or VI. In the latter, however, either the synthesis or the maturation of the cortex appears to be deficient.

It is difficult to deduce from the available data the exact correlation between the morphological stages and the chemical resistances, since the sporulating populations are heterogeneous and electron microscope studies examine 5 to 100% of the cell population while the chemical resistances can only be measured with precision in less than 10% of the population. It should be noted, however, that in two mutants, FA10 and FE9, one-third of the cells reach stage VI while only about 1% become resistant to chloroform and 0.1% to heat. This observation can be explained in two ways: (i) although the cells appear to be at stage VI, one of the spore envelopes is abnormal, or (ii) the chemical resistances require the synthesis of some sporulation-specific product(s) in addition to the presence of normal spore envelopes. [This product cannot be DPA since DPA-less spores are chloroform-resistant (Balassa *et al.*, 1979).]

One of the decadent mutations, *SpoL1*, was localized by transduction in the *lys-phe* region between *aroD* and *acf*. It is linked by transformation to *aroD*. This region is very rich in sporulation loci and contains a large number of late sporulation mutations blocked at stages III, IV or V (Piggot & Coote, 1976). In the absence of fine structure mapping and of more detailed phenotypic studies it is difficult to decide whether one of these loci is identical with *SpoL1* or if the latter represents a new locus. The other decadent mutants have not yet been mapped but some of them present a phenotype sufficiently different from *SpoL1* to suppose that they correspond to one or more other loci.

The phenotype of the decadent mutants can be tentatively described as follows: during sporulation, each switch in the morphogenetic sequence occurs more slowly and with a lower probability than in the *Spo*⁺ cells. The synthesis of extracellular protease, an early sporulation product, is apparently unaffected by decadent mutations while late events such as the synthesis of DPA and lysis of the sporangium do not take place. (Unfortunately biochemical events associated with intermediate stages were not measured.)

Sporulation is controlled by sequential gene activation (Balassa, 1971; Piggot & Coote, 1976). The characteristics of the decadent mutations suggest that they are implicated in the quantitative control of sporulation. Their pleiotropic effects make it very unlikely that they involve a structural gene for a spore-specific product. Since the decadence is, in general, extended over at least three morphological stages, it is difficult to imagine that the quantities of a single sporulation-specific product would be affected.

The phenotype of decadent mutations can be explained if impaired function of a sporulation gene leads to only partial activation of the next gene in the sequence, which in turn induces the next gene only in a fraction of the cells and even there only to a lower level, and so on. This would result in a progressive decrease of what one could call the 'flux of induction' and, as a consequence, in a low probability of switch at all the steps of the sporulation sequence (Balassa & Yamamoto, 1970; Balassa, 1971). Some of the spore control (*Sco*) mutations also appear to affect the switch mechanism specifically (Balassa *et al.*, 1974;

Sousa *et al.*, 1978). They differ from the decadent mutations in the overproduction of sporulation-associated proteins and in the fact that although the switch from one morphological stage to another is slowed down in these mutants, it affects all cells and, at the end of the process, most of them reach stage VI and heat resistance.

Other explanations which do not directly involve sequential gene activation are also possible. Thus, decadent mutants could be leaky for the production of a single gene product, necessary for all the specific events during a long period of the sporulation process or involved in energy production during sporulation. On the other hand, the simultaneous presence of two or more oligosporogenous mutations could lead to a phenotype close to that of the decadent mutants (Coote & Mandelstam, 1973). This is not the case for DB16 and for FE9. For the other decadent mutants obtained by nitrosoguanidine-induced mutagenesis, the presence of multiple mutations cannot be excluded. If this were the case, however, one would expect two distinct, clearly defined blocks in the sporulation process, instead of the progressive 'decadence' observed over several morphological stages.

In conclusion, we suggest that the decadent mutations are single pleiotropic mutations which cause stepwise decrease of the activity of a whole set of sporulation genes. Further genetic and biochemical studies are necessary to confirm this hypothesis.

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